JOURNAL OF VIROLOGY, Aug. 2002, p. 8218–8224 Vol. 76, No. 16
0022-538X/02/$04.00+0 DOI: 10.1128/JVI.76.16.8218–8224.2002

Characterization of a Polytropic Murine Leukemia Virus Proviral Sequence Associated with the Virus Resistance Gene *Rmcf* of DBA/2 Mice

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Received 8 March 2002/Accepted 10 May 2002

The DBA/2 mouse *Rmcf* gene is responsible for in vivo and in vitro resistance to infection by the polytropic mink cell focus-forming (MCF) virus subgroup of murine leukemia viruses (MLVs). Previous studies suggested that *Rmcf* resistance is mediated by expression of an interfering MCF MLV envelope (Env) gene. To characterize this *env* gene, we examined resistance in crosses between *Rmcf*^{r} DBA/2 mice and *Mus castaneus*, a species that lacks endogenous MCF *env* sequences. In backcross progeny, inheritance of *Rmcf* resistance correlated with inheritance of a specific endogenous MCF virus *env*-containing 4.6-kb *Eco*RI fragment. This fragment was present in the DBA/2N substrain with *Rmcf*-mediated resistance but not in virus-susceptible DBA/2J substrain mice. This fragment contains a provirus with a 5′ long terminal repeat and the 5′ half of *env*; the *gag* and *pol* genes have been partially deleted. The Env sequence is identical to that of a highly immunogenic viral glycoprotein expressed in the DBA/2 cell line L5178Y and closely resembles the *env* genes of modified polytropic proviruses. The coding sequence for the full-length *Rmcf* Env surface subunit was amplified from DNAs from virus-resistant backcross mice and was cloned into an expression vector. NIH 3T3 and BALB 3T3 cells stably transfected with this construct showed significant resistance to infection by MCF MLV but not by amphotropic MLV. This study identifies an *Rmcf*-linked MCF provirus and indicates that, like the ecotropic virus resistance gene *Fv4*, *Rmcf* may mediate resistance through an interference mechanism.

Numerous mouse chromosomal genes are responsible for resistance to murine leukemia viruses (MLVs). While many of these genes mediate the immunological response, others interfere directly with virus infection and replication. Many of the latter genes affect the virus-receptor interaction, in some cases because they encode functional variants of the cell surface viral receptors. At least three such functionally variant receptors in mice have been described. The resistance of *Mus dunni* cells to Moloney ecotropic virus is due to a polymorphism of mCAT1 receptor gene *Slc7a1* (5). There are also two known polymorphisms of polytropic/xenotropic receptor gene *Xpr1*. One of these, termed *Sxv*, is responsible for resistance of cells of the common inbred strains to xenotropic virus (14), and a second polymorphism, found in *Mus castaneus*, is responsible for resistance to both polytropic and xenotropic MLVs (17, 19).

A second group of receptor-mediated resistance genes is thought to function primarily through an interference mechanism. The best-characterized of these genes, *Fv4*, encodes an ecotropic envelope (Env) glycoprotein, expression of which is thought to interfere with receptor binding of exogenous ecotropic virus (12, 30). Studies on another gene, the *Rmcf* resistance locus of DBA/2 mice, suggest that an analogous interference mechanism may be responsible for *Rmcf*-mediated resistance to the polytropic mink cell focus-forming (MCF) MLVs.

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The resistance of DBA/2 strain mice to MCF virus was first described by Ruscetti and her colleagues (24), who noted that these mice endogenously express an MCF virus Env glycoprotein and do not produce MCF virus following inoculation with Friend ecotropic MLV. Inhibitors of glycoprotein synthesis reduce expression of the MCF-related Env glycoprotein and also reduce resistance to exogenous infection (1). Hartley and her colleagues (10) showed that this resistance is due to a single gene, which they termed *Rmcf*, on chromosome 5 that reduces virus titers in vitro by 10- to 100-fold. Subsequent studies demonstrated that this gene also increases the latency or incidence of diseases normally mediated by the emergence of recombinant MCF viruses (3, 25).

Identification and characterization of the *Rmcf*-associated Env glycoprotein and the proviral sequences responsible for its expression have been difficult. DBA/2 mice, like other inbred strains, contain multiple proviral copies of the MCF viral *env* sequence. Although many of these proviruses have been positioned on the mouse linkage map (7), none of the mapped DBA/2 proviruses was identified as a candidate for *Rmcf*.

In this study, we mated DBA/2 mice with a wild mouse species that lacks endogenous MCF virus-related Env sequences in order to reduce the number of MCF proviruses present in the genomes of mice with *Rmcf*-mediated resistance. We identified a specific MCF virus-related provirus associated with inheritance of *Rmcf*-mediated resistance, and we cloned and characterized this gene.

MATERIALS AND METHODS

Mice. CAST/Ei mice (CAST) and DBA/2J mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). DBA/2N mice were obtained from the Small Animal Section, National Institutes of Health (Bethesda, Md.). CAST

males and females were bred to DBA/2 mice, and the F_1 hybrids were crossed with CAST to produce first-backcross mice (CDC cross).

Genetic typing. Genomic DNA was prepared by standard methods from liver, from tail biopsy samples, or from cells cultured from tail biopsy samples. PCR amplification of simple sequence repeats was carried out as described previously (4). Simple sequence repeat marker *D1Mit33* was selected for monitoring the inheritance of the chromosome 1 region containing *Xpr1* (29), and *D5Mit1* and *D5Mit3* were used as linked markers for *Rmcf* (17). PCR products were fractionated on 3% MetaPhor agarose (BioWhittaker Molecular Applications) gels stained with ethidium bromide. Individual backcross progeny were tested; a pooling strategy was not feasible since amplification was often less efficient with the CAST alleles.

Viruses, cells, and virus assays. The viruses used in the infectivity assays were originally obtained from J. Hartley (National Institute of Allergy and Infectious Diseases, Bethesda, Md.). For these experiments, polytropic virus Moloney MCF-HIX (6) and amphotropic virus 4070A (9) were used.

Susceptibility of individual mice to MCF virus was tested by infecting cultures of tail biopsy tissue prepared as described by Lander and her colleagues (16). Briefly, tail tips of 7- to 10-day-old mice were minced and incubated for 45 min with 2 ml of collagenase (150 U/ml). Cells from this suspension were cultured in Dulbecco's medium with 10% fetal calf serum and antibiotics. When the cultures reached confluence, the cells were passaged and infected 1 day later with dilutions of polytropic and amphotropic virus in the presence of Polybrene (4 μg/ml; Aldrich, Milwaukee, Wis.). After 4 to 5 days, cultures were UV irradiated and overlaid with 6×10^5 mink S^+L^- cells (22). Foci were counted 6 to 7 days later.

DNA extraction and Southern blotting. DNA extracted from livers of adult mice was digested with various restriction enzymes according to the manufacturers' suggestions, separated on 0.4% agarose gels, and transferred to nylon membranes (Hybond N+; Amersham, Piscataway, N.J.). Filters were hybridized with radiolabeled proviral fragments. These hybridization probes included the MCF and xenotropic *env*-specific segments described by O'Neill and his colleagues (21). Other hybridization probes were designed in the course of these studies.

Cloning of the *Rmcf*-associated provirus. Genomic DNA of DBA/2N mice was digested to completion with *Eco*RI and separated on a 0.6% agarose gel. DNA enriched for fragments of about 4.6 kb was gel purified with a gel extraction kit (Qiagen, Valencia, Calif.). The provirus was cloned by using GenomeWalker (Clontech, Palo Alto, Calif.). The DNA was ligated to oligonucleotide *Eco*RI adapters (R12-mer, 5′-AATTCTCGGTGA-3′; R24-mer, 5′-AGCACTCTCCA GCCTCTCACCGAG-3′) in a 60-μl reaction mixture containing 2 μl of T4 DNA ligase. The ligation reaction mixture was incubated at 16°C overnight.

PCR was used to amplify segments of the gel-purified *Rmcf*-associated proviral sequence (Fig. 1). The PCR mixture (20 μl, final volume) contained 0.5 μg of the ligation mixture, 2.5 U of Ampli*Taq* Gold DNA polymerase (PE Applied Biosystems, Foster City, Calif.), and 20 pmol of the adapter primers and MCF virus-specific primers. The sequences of the MCF virus-specific primers (Fig. 1B) are as follows: UE1, 5′-GGATACACGCCGCTCACGTA-3′ (MCF virus *env*-specific forward primer); INa, 5′-CAAGCATGAGACTGCCAAAG-3′ (MCF virus *pol*-specific forward primer); INb, 5′-ATAGAGACCTCAACCCCGTA-3′ (MCF virus *pol*-specific forward primer); INr, 5′-CTTTGGCAGTCTCATGCT TG-3′ (MCF virus *pol*-specific reverse primer).

The PCRs were carried out in a GeneAmp PCR system 9700 machine (PE Applied Biosystems). The reactions were performed for 35 cycles with a 30-s DNA denaturation step at 95°C, a 30-s annealing step, and a 1-min extension step at 72°C. The annealing temperature in the first cycle, 63°C, was subsequently reduced by 1°C each cycle for the next 8 cycles and was then maintained at 55°C for the remaining 27 cycles.

The two overlapping PCR products derived from the 3′ end of the 4.6-kb fragment (UE1-R24, 623 bp; INb-R24, 1.7 kb) (Fig. 1B) were cloned into pCR2.1-TOPO (Invitrogen Co., Purchase, N.Y.) and sequenced. No R24-INr PCR product was obtained. The INb-R24 sequence revealed a *Hin*dIII restriction site located 500 bp from the 5′ end. A phage library was then constructed to clone the 5′ *Eco*RI-*Hin*dIII end of this 4.6-kb fragment. Gel-purified DBA/2N *Eco*RI fragments of about 4.6 kb were digested with *Hin*dIII. The DNA was then ligated into the *Eco*RI-*Hin*dIII cloning sites of the lambda SCREEN vector (Novagen, Madison, Wis.). The library was screened with a 330-bp hybridization probe (Pol probe; Fig. 1) derived from the 5′ end of the integrase gene contained within the sequenced INb-R24 PCR product. Positive recombinant phages were isolated, autosubcloned by Cre-mediated plasmid excision, and sequenced with the SP6 primers.

Sequence analysis. Nucleotide sequences of all PCR products and phage library clones and inferred amino sequences were analyzed with the MacVector, version 6.5 (Genetics Computer Group, Madison, Wis.), computer program.

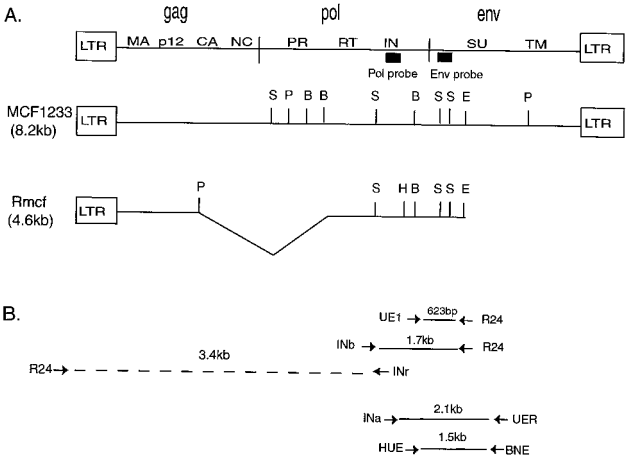

FIG. 1. Structure of the *Rmcf*-associated provirus. (A) Below the general structure of an integrated MLV genome are abbreviated restriction maps for prototypical polytropic virus MCF1233 and for the *Rmcf* provirus contained within the genomic *Eco*RI fragment. Black boxes, Env and Pol hybridization probes. Restriction sites: P, *Pst*I; S, *Sma*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. (B) PCR primers and products. The dashed line indicates that no product was obtained with the R24 and INr primers.

Sequence homology searches of GenBank were conducted using both BLASTn and BLASTx.

Transfection and interference assay. To obtain a full-length *Rmcf* envelope SU sequence to evaluate for interference, PCR was performed using as the template a pool of DNAs from *Rmcf*-positive backcross mice of the CDC cross and as primers integrase forward primer INa and reverse universal polytropic/xenotropic primer UER from the 5′end of the TM region (Fig. 1). The UER primer represents nucleotides 1456 to 1476 of GenBank sequence MUSMLVPB (accession no. M17327), and its sequence is 5′-TGGAGCTGCTCGAATTGCT TG-3′.

This full-length *Rmcf* SU was subcloned into expression plasmid vector pcDNA3.1(+) (Invitrogen, Frederick, Md.) by using a forward primer, HUE, specific to the 5′ end of *Rmcf* and a reverse primer, BNE, based on the coding sequence of the end of the SU envelope protein. *Hin*dIII and *Bam*HI restriction sites were introduced into the HUE and BNE primers, respectively. The nucleotide sequences of the primers are as follows: HUE, 5′-ACGCAAGCTTCACG CCGCTCACGTAAAAGC-3′; BNE, 5′-ACAGTGGATCCTTATCTTTTATA TTTGGTTT-3′.

To investigate the effect of *Rmcf* SU gene expression on MCF MLV infectivity, NIH 3T3 and BALB 3T3 cells expressing the *Rmcf* SU were generated by transfection of the *Rmcf* SU DNA subcloned into the pcDNA3.1(+) mammalian expression vector. Transfection was accomplished with the Qiagen SuperFect transfection kit. The transfected cells were selected with antibiotic G-418 (1.2 mg/ml) and cloned by limiting dilution. The transfected cell lines were examined for expression of *Rmcf* SU gene transcripts by reverse transcription-PCR (RT-PCR) using vector primers complementary to the sequences upstream and downstream of the vector cloning site.

LacZ pseudotype virus was generated by transfection of TELCeB6 cells with polytropic MCF247 envelope expression vector pCRUCM and amphotropic 4070A envelope expression vector pCRUCA, kindly provided by J.-L. Battini (Institute Pasteur, Paris, France). TELCeB6 produces noninfectious viral particles harboring the MFGnlslacZ retrovirus vector. The stably transfected cell lines expressing *Rmcf* were infected with the LacZ(MCF247) and LacZ(4070A) pseudotype virus. Two days after infection, cells were fixed with 0.5% glutaraldehyde and stained to reveal the presence of β-galactosidase activity. Infectious titers were expressed as the number of blue CFU per milliliter of virus supernatant.

RESULTS

Inheritance of *Rmcf* resistance in crosses between DBA/2 and *M. castaneus*. Identification of the proviral *env* gene responsible for the expression of the interfering polytropic en-

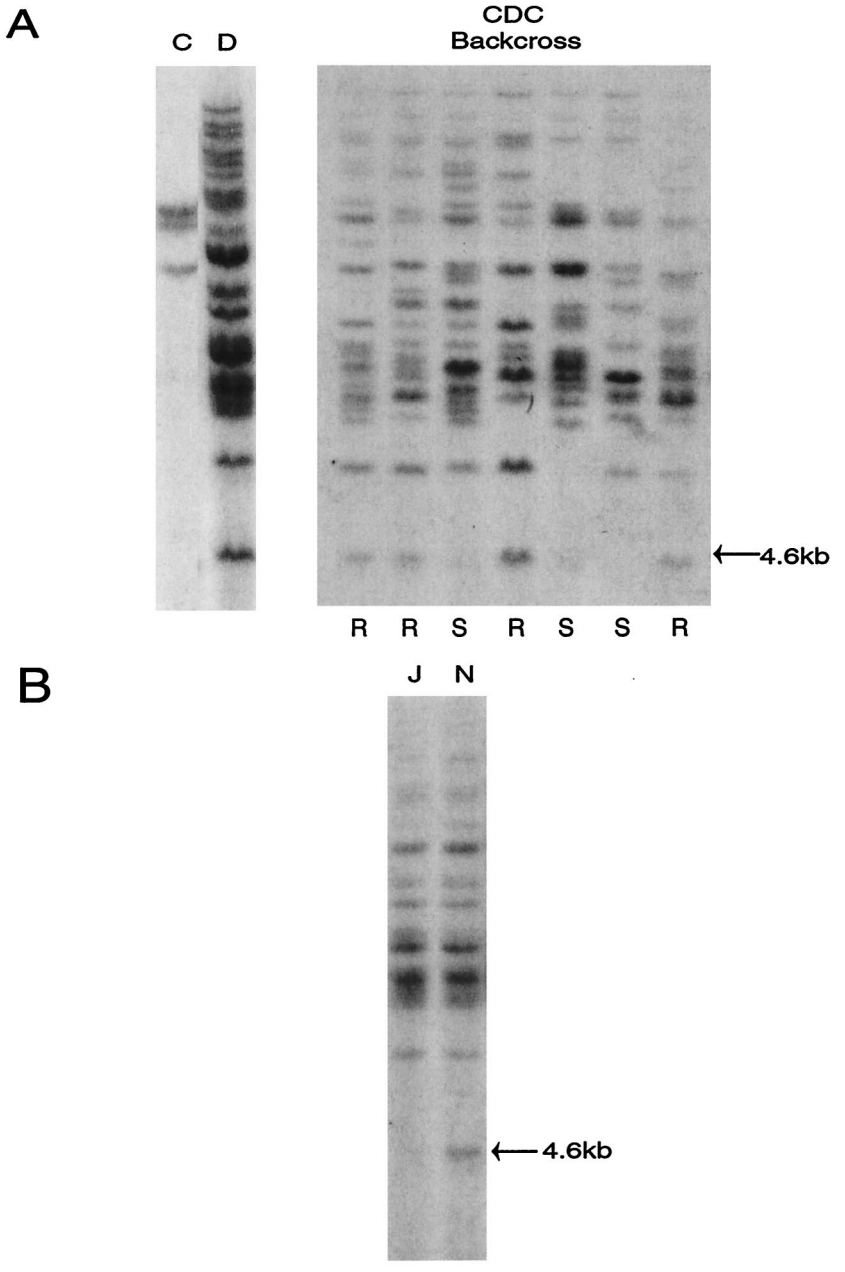

FIG. 2. Southern blot analysis of DNAs from parental strains and progeny of the CDC backcross. (A) *Eco*RI digests of the indicated DNAs were hybridized with the MCF-specific Env probe. C, CAST; D, DBA/2N; CDC backcross, seven individual backcross mice. Backcross mice typed as resistant (R) and sensitive (S) are indicated. (B) *Eco*RI digests of DBA/2J (J) and DBA/2N (N) hybridized with MCF *env*.

velope glycoprotein in DBA/2 mice is complicated by the fact that these mice contain numerous copies of polytropic MLV *env* sequences (Fig. 2A). In contrast, some species of wild mice contain few polytropic proviral *env* genes (15). Therefore, we used classical genetic crosses between DBA/2 and a wild mouse parent to reduce the number of these proviruses in mice with the resistance phenotype. *M. castaneus* was selected as the wild mouse parent because, as shown previously, *M. castaneus* contains only three copies of the MCF virus *env* sequence (Fig. 2A).

Progeny of this CDC backcross were typed for resistance to MCF virus by titering virus on cultured tail biopsy tissue of individual mice. All mice were tested in duplicate, and each set of cultures was also infected with amphotropic virus as a positive control. Sample results for a single litter are shown in Table 1. At the virus dilutions used, little or no virus was detected in cells scored as resistant.

Two factors complicated the analysis of this cross. First, as reported in earlier studies (17, 18), about 75% (not 50%) of the CDC backcross mice were resistant to MCF infection. This is consistent with the inheritance of *Rmcf* together with a second, unlinked resistance gene. This additional gene was

TABLE 1. Susceptibility to polytropic MCF MLV of individual mice in a single litter of the CDC backcross[a]

Mouse	Genotype for:		No. of foci	Susceptibility[b]
	D5Mit1 (*Rmcf*)	*D1Mit33* (*Xpr1*)		
637-1	CD	CD	16	R
637-2	CD	CD	6	R
637-3	CD	CC	0	R
637-4	CD	CC	0	R
637-5	CC	CD	>700	S
637-6	CC	CC	0	R

[a] CAST and DBA alleles of the marker loci *D5Mit1* and *D5Mit33* are designated C and D, respectively. Mice inheriting the DBA allele at *Rmcf* ($D5Mit1^{CD}$) and both CAST alleles at *Xpr1* ($D1Mit33^{CC}$) are expected to be resistant to infection. Cultured tail cells from individual mice were infected with MCF MLV and 4 days later were irradiated and overlaid with mink S^+L^- cells.
[b] R, resistant; S, susceptible.

determined to be a novel recessive resistance and was mapped to the locus encoding the MCF virus cell surface receptor, *Xpr1* (17). CDC mice homozygous for M. *castaneus Xpr1* were thus resistant to virus infection and could not be typed for *Rmcf*-mediated resistance. Therefore, *Rmcf*-mediated resistance could only be reliably typed in one-half of the progeny, the half that inherited one functional copy of the *Xpr1* receptor gene from the DBA/2 parent.

A second complication in typing the CDC cross was the fact that different results were obtained depending on the DBA/2 substrain used for breeding: DBA/2N or DBA/2J (Table 2). Resistance showed two-gene inheritance in the DBA/2N cross as described above; 154 of the 208 progeny (or 74.0%) were typed as resistant ($\chi^2 = 0.10$, $P < 0.8$). In contrast, in the DBA/2J cross, resistance showed single-gene inheritance; only 47 of 101 mice were resistant, or 46.5% ($\chi^2 = 0.49$, $P < 0.5$). Progeny of both crosses were typed for markers closely linked to *Xpr1* and to *Rmcf*. Results indicated that resistance could be attributed to both genes in the DBA/2N progeny but only to the CAST *Xpr1* gene in the DBA/2J cross (Table 2). These results indicated that the DBA/2J parental mice used for the cross did not have *Rmcf*-mediated resistance.

Identification of an *Rmcf*-associated proviral *env* gene. The proviral contents of parental DBA/2N and CAST mice are shown in Fig. 2. *Eco*RI digestion reveals only three MCF *env* fragments in CAST mice and multiple copies in DBA/2. Seventy-five of the 208 backcross mice that had been tested by tail biopsy for susceptibility to MCF virus and for genetic markers linked to *Rmcf* and *Xpr1* were sacrificed, and livers were extracted for high-molecular-weight DNA. Southern blot analysis of these DNAs showed these backcross mice to have one-half the number of MCF *env* genes of the DBA/2 parent (Fig. 2). Inheritance of *Rmcf*-linked marker *D5Mit1* correlated with inheritance of a single unique *Eco*RI MCF-related *env* gene of 4.6 kb ($r = 3/75$; recombinational distance = 4.0 ± 2.3). Of these 76 mice, 32 were resistant to MCF virus because they were homozygous for the defective CAST *Xpr1* receptor. Of the remaining 44 mice, 17 were typed as resistant and all 17 inherited this 4.6-kb *env*-reactive fragment. This band was missing in 25 of the 27 susceptible mice. The two discrepancies were littermates that were tested for virus susceptibility at the same time. They were typed as heterozygous for both markers flanking *Rmcf*, *D5Mit1* and *D5Mit3*, suggesting the possibility of an error in the virus assays. The results of this backcross analysis identify a specific proviral *env* sequence at or very near *Rmcf*.

TABLE 2. Genetic control of resistance to polytropic MCF MLVs in progeny of the CDC backcrosses generated using DBA/2N and DBA/2J as the parental strains

Genotype[a] for:		No. of backcross mice of strain:			
		DBA/2J		DBA/2N	
D5Mit1 (*Rmcf*)	*D1Mit33* (*Xpr1*)	Resistant	Susceptible	Resistant	Susceptible
CC	**CC**	27	1	50	0
CD	**CC**	16	0	52	0
CC	CD	2	24	1	52
CD	CD	2	30	51	2

[a] CAST and DBA alleles at the two marker loci are designated C and D, respectively. Genotypes expected to produce resistance are in boldface.

Further evidence for the *Rmcf* association of this provirus comes from the comparison of DBA/2N and DBA/2J mice. As indicated above, only DBA/2N crosses show *Rmcf*-mediated resistance. Southern analysis of DBA/2N mice with *Rmcf*-mediated resistance and virus-sensitive DBA/2J mice showed that the two substrains appeared identical to one another in proviral content except that the 4.6-kb *Eco*RI *env* fragment was present only in DBA/2N mice. This further establishes the association between this provirus and resistance. Based on these observations we cloned and characterized the 4.6-kb *Eco*RI fragment.

Cloning and sequence analysis of the 4.6-kb *Eco*RI fragment. DNA of DBA/2N was digested with *Eco*RI and electrophoresed through a 0.6% agarose gel. Fractions of DNA in the 4.6-kb size range were purified. Repeated attempts to clone the *Eco*RI fragment in λ gt10 (Stratagene, La Jolla, Calif.) were unsuccessful. Instead, *Eco*RI adapters were ligated to the ends of the purified DNA fragments to generate template DNA for the specific amplification of unknown DNA fragments by adapter PCR. Because this fragment contains the MCF virus *env* sequence, as shown by Southern blot analysis, MCF *env*- and *pol*-specific primers were used together with the adapter primers to try to separately amplify the 5′ and 3′ ends of the *env*-containing 4.6-kb fragment. Products of 623 bp and 1.7 kb from the 3′ end were successfully amplified using, respectively, UE1 and INb as the forward primers and the R24 adapter reverse primer (Fig. 1). However, the larger 3.4-kb 5′ end could not be amplified by using as primers the adapter forward primer (R24) and the MCF virus *pol* reverse primer (INr).

To obtain the 5′ end of this fragment, a phage library was constructed. Sequence analysis of the 3′-end PCR product, INb-R24, identified a *Hin*dIII site 500 bp from the 5′ end; this was confirmed by Southern blotting using a 330-bp probe derived from the integrase gene sequence from the 3′ end of the 4.6-kb fragment (Pol probe; Fig. 1). The 4.6-kb gel-purified *Eco*RI fragments from DBA/2N were digested with *Hin*dIII and cloned into the lambda SCREEN vector (Novagen). The library was screened with the Pol probe, and three clones were selected for sequencing.

Sequence analysis. Nucleotide sequence analysis shows that the 4.6-kb fragment comprises 4,668 bp (GenBank accession no. AF490352) and shows high sequence homology with full-

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        ┌Signal Peptide                    ┌gp70
Rmcf    MEGPAFSKPLKDKINPWAPLIVLGILIRAGVSVPHDSPHQVFNVTWRVTNL
MX33    ----------------------------------------------------
CD15-7  ----------------------------------------------------
MCF247  ----------------G---------------R-----------------

Rmcf    MTGQTANATSLLGTMTDAFPKLYFDLCDLIGDDWDETGLGCRTPGGRKRAR
MX33    ----------------------------------------------------
CD15-7  ----------------------------------------------------
MCF247  ----------------------------------------------------

Rmcf    TFDFYVCPGHTVPTGCGGPREGYCGKWGCETTGQAYWKPSSSWDLISLKRG
MX33    ----------------------------------------------------
CD15-7  ----------------------------------------------------
MCF247  ----------------------------------------------------

Rmcf    NTPRNQGPCYDSSAVSSGIQGATPGGRCNPLVLEFTDAGKKASWDGPKVWG
MX33    ----------------------------------------------------
CD15-7  ----------------------------------------------------
MCF247  ---Q-------------D-K-------------------------------

Rmcf    LRLYRSTGTDPVTRFSLTRQVLNIGPRIPIGPNPVITDQLPPSRPVQIMLP
MX33    ----------------------------------------------------
CD15-7  ----------------------------------------------------
MCF247  --------I-------------------V-----------------------

Rmcf    RPPQPSPTGAASI*********QPGTGDRLLNLVDGAYQALNLTSPDKTQE
MX33    ----------------------------------------------------
CD15-7  ----------------------------------------------------
MCF247  -----P-P----TVPETAPPSQ------------K-----------------

Rmcf    CWLCLVSGPPYYEGVAVLGTYSNHTSAPANCSVASQHKLTLSEVTGQGLCV
MX33    ----------------------------------------------------
CD15-7  ----------------------------------------------------
MCF247  ----------------------------------------------------I

Rmcf    GAVPKTHQALCNTTQNTSDGSYYLAAPAGTIWACNTGLTPCLSTTVLNLTT
MX33    ----------------------------------------------------
CD15-7  ----------------------------------------------------
MCF247  ---------------K-----------T--T---S------I---I-D---

Rmcf    DYCVLVELWPKVTYHSPGYVYGQFERKTKYKR
MX33    --------------------------------
CD15-7  --------------------------------
MCF247  ----------R------S---H----RA----
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FIG. 3. Amino acid sequence encoded by the 5′ end of the *Rmcf* SU. The sequence is compared with those of prototypical modified polytropic provirus Mx33 (28), the expressed SU found in L5178Y DBA/2 cells (8), and MCF247 (11).

length polytropic MLV clone MCF1233 (GenBank accession no. U13766) (27). Relative to MCF1233, however, there is a large deletion of approximately 2.2 kb in the 4.6-kb clone that extends from the capsid (CA) gene of Gag to the 3′ region of Pol within the RNase H domain (Fig. 1). The 5′ end of this 4.6-kb fragment contains only 8 bp of flanking cellular DNA.

Like other MCF virus *env* genes, the *Rmcf env* is cleaved by *Eco*RI just 3′ to the coding sequence for the proline-rich region, and therefore the sequenced clone contains only the 5′ half of *env*. This truncated *Rmcf env*, from the end of *pol* to the *Eco*RI site in the 3′ end, contains the complete receptor binding domain. This 623-bp region has 99% nucleotide sequence homology with two previously described MLV *env* sequences, that of the Mx33 modified polytropic provirus of HRS mice (GenBank accession no. M17327) (28) and that of the gene encoding the viral Env glycoprotein responsible for the high immunogenicity of DBA/2 cell line L5178Y (GenBank accession no. S77015); the encoded amino acid sequences are 100% homologous (8) (Fig. 3). Like these previously described *env* genes, the *Rmcf env* contains a 27-bp deletion within the coding sequence for the proline-rich region of SU.

As shown in Fig. 1, a complete long terminal repeat (LTR; U3, R, U5) was found at the 5′ end of the *Rmcf* provirus. The U3 region is virtually identical to that of the Mx33 provirus, with only a single nucleotide substitution. This U3 region contains the 190-bp insert characteristic of LTRs associated with polytropic proviruses. The remaining segments of the *Rmcf* provirus, R, U5, the 5′ untranslated region, the Gag MA, p12, and CA genes, and the truncated *pol* region encoding integrase also show substantial homology with numerous previously described endogenous proviral sequences and those of infectious polytropic MLVs such as MCF1233 (not shown).

Transfection and interference assay. The original 4.6-kb *Rmcf* provirus contained the coding sequence for a partial SU truncated by an *Eco*RI site. Attempts to use other restriction enzymes to identify an *Rmcf*-derived proviral fragment containing the 3′ *env* and LTR sequences were not successful due to the presence of the numerous *env*-related fragments remaining in the first backcross mice. Breeding problems with CAST mice prevented the generation of additional backcross generations to reduce the background of MCF *env* genes. Therefore, for functional assays, the coding sequence for full-length SU was amplified from *Rmcf*-positive backcross mice with the INa and UER primers. This 2.1-kb PCR product could not be amplified from backcross mice lacking *Rmcf*. As expected, the nucleotide sequence of the 5′ end of this 2.1-kb product (GenBank accession no. AF490353) has 100% identity with the corresponding region of the 4.6-kb *Eco*RI fragment. The nucleotide sequence from the *Eco*RI site of *env* to the 3′ end of *env* has 99% nucleotide identity with that of the Mx33 modified polytropic provirus, and the encoded amino acid sequences have 100% identity; this *env* nucleotide sequence also has 100% nucleotide identity with the coding sequence for the viral glycoprotein of DBA/2 cell line L5178Y (GenBank accession no. S77015) (Fig. 3) (8).

We then designed sense (HUE) and antisense (BNE) primers to sequences in the 2.1-kb PCR product to amplify just the *Rmcf* SU (Fig. 1), with the 2.1-kb PCR product as the template. This 1.5-kb PCR fragment was subjected to sequential digestion with *Hin*dIII and *Bam*HI before purification and cloning into expression vector pcDNA3.1.

A pseudotyping assay was used to test for the interference properties of the *Rmcf* SU. The expression vector containing the *Rmcf* SU was transfected into NIH 3T3 and BALB 3T3 cells. Expression of the *Rmcf* SU in the transfected lines was confirmed by RT-PCR using vector primers (sense, 5′-TAAT ACGACTCACTATAGGG-3′; antisense, 5′-TAGAAGGCA CAGTCGAGG-3′); transfected lines produced the expected 1.5-kb RT-PCR product, but this product was absent from untransfected cells and cells transfected with the vector alone. The transfected cells were then tested for susceptibility to β-galactosidase-encoding LacZ(MCF247) and LacZ(4070A) pseudotype virus (Table 3). NIH 3T3 and BALB 3T3 cells transfected with the pcDNA3.1 vector alone were highly susceptible to infection by LacZ(MCF247) pseudotype virus, whereas cells transfected with pcDNA3.1-*Rmcf* SU showed reduced sensitivity to LacZ(MCF247) pseudotype virus. Infectivity with amphotropic LacZ(4070A) virus was not altered by expression of *Rmcf* SU. These results suggest that the *Rmcf* SU can inhibit the entry of exogenous MCF MLV, consistent with the proposed interference mechanism of *Rmcf* resistance.

TABLE 3. NIH 3T3 and BALB 3T3 cells transfected with *Rmcf* expression vector are partially resistant to polytropic MLV infection

Target cell	Titer of LacZ pseudotype (CFU/ml) for:	
	MLV-M	MLV-A
NIH 3T3	1.0×10^6	7.0×10^5
NIH 3T3(C15)[a]	1.2×10^4	9.4×10^5
BALB 3T3	2.1×10^5	2.2×10^4
BALB 3T3(C4)[a]	1.6×10^3	1.7×10^4

[a] G-418-resistant clone transfected with *Rmcf*DNA.

DISCUSSION

This study used a classical genetic approach to reduce the MCF MLV proviral content of DBA/2 mice with *Rmcf*-mediated resistance in order to permit identification of a specific provirus associated with *Rmcf*-mediated resistance. The relationship between this provirus and virus resistance was established by several lines of evidence. First, the provirus was mapped to the region of chromosome 5 containing the *Rmcf* locus, and inheritance of this provirus correlated with inheritance of the resistance phenotype. Second, the provirus is carried by virus-resistant DBA/2N substrain mice but not by virus-sensitive DBA/2J substrain mice. Third, expression of the *Rmcf* SU in cultured cells resulted in partial resistance to infection with MCF but not amphotropic MLVs. Fourth, expression of an SU identical to the *Rmcf*-encoded Env sequence has been reported only once previously, in a cell line of DBA/2 origin (8). Taken together, these results support previous proposals (1, 24) that *Rmcf* functions by controlling production of a viral Env glycoprotein that interferes with exogenous virus infection.

The absence of *Rmcf*-mediated resistance in our DBA/2J crosses was surprising since the reports that first described *Rmcf* in the early 1980s identified this resistance in both DBA/2J (10) and DBA/2N (24). The DBA/2J mice we used here clearly lacked this provirus. Excision of proviruses is rare, but, interestingly, one of the best-documented examples of this phenomenon is the deletion of the ecotropic provirus from DBA/2 strain mice (26). While our data suggest that the *Rmcf* provirus may have been lost from the DBA/2J strain in the last 20 years of breeding, we did not attempt to document changes over time in the DBA/2J strain, nor did we extend these studies to type additional samples from other DBA substrains or from other strains with *Rmcf*-mediated resistance such as CBA/N.

The *Rmcf* provirus shows some structural resemblance to the provirus integrated at ecotropic virus resistance gene *Fv4* (12) in that both contain an intact *env* and 3′ LTR and both have deletions that remove a substantial portion of *pol*. Unlike *Rmcf*, however, *Fv4* lacks *gag* sequences and a 5′ LTR and therefore uses a cellular promoter for expression (H. Ikeda, unpublished observations). This difference may account for the fact that *Rmcf*-mediated resistance is reliably detected in vitro and in vivo, whereas cultured cells from *Fv4*-positive mice show variable levels of virus resistance (32). While there are differences in the transcriptional regulation of these two resistance genes, it seems likely that these genes have a common mechanism of resistance. This mechanism is based on interference, although for *Fv4* the detailed description of the processes that specifically produce resistance is still under active investigation (30, 33).

The nucleotide sequence of the *Rmcf* provirus shows no obvious features that could account for its unique association with resistance among the numerous nonecotropic proviruses found in laboratory mouse strains. The LTR sequence is unremarkable. The *Rmcf env* sequence closely resembles that of prototypical modified polytropic provirus Mx33. Like sequences of these modified polytropic proviruses, the *Rmcf* sequence has a 27-bp deletion within the coding sequence for the proline-rich region of SU. The impact of this deletion on virus replication and infectivity has not been formally examined, although few viruses or viral proteins with this deletion have been identified, e.g., the Env glycoprotein of DBA L5178Y lymphoma cells (8) and a single Friend virus isolate that is infectious but not pathogenic (13). While other studies have shown that substantial deletions introduced into this SU region in ecotropic MLVs result in altered infectivity and Env protein processing (31), this suggests no obvious mechanism for resistance involving the 27-bp *env* deletion.

Fv4 and *Rmcf* are not the only genes for which virus resistance is mediated by expression of endogenous *env* copies. In other studies of *Fv4*-positive wild mouse species *M. castaneus*, we identified another virus resistance gene, which we now term *Rmcf2* (18). This gene blocks infection by MCF virus, and its locus, like those of *Fv4* and *Rmcf*, contains an expressed proviral *env* (T. Wu and C. A. Kozak, unpublished data). The fact that mice have recruited proviral sequences to provide protection against exogenous infection on at least three separate occasions (*Fv4*, *Rmcf*, and *Rmcf2*) suggests that this is not an unusual phenomenon and may, in fact, be important in mouse populations such as *M. castaneus* whose survival is dependent on adaptation to widespread endemic infections. This type of resistance gene is also not unique to mice. Comparable genes in chickens (23) and cats (20) have been described, and it should be noted that the mouse *Fv1* gene also represents a retrovirus *gag*-related sequence, although its mechanism of resistance remains unknown (2). Further studies on these resistance genes may be useful in describing adaptations to infection as well as elucidating the mechanisms of retrovirus interference and receptor usage.

ACKNOWLEDGMENTS

We acknowledge the expert technical assistance of M. Charlene Adamson (deceased). We thank Caroline Ball for editorial assistance in the preparation of the manuscript.

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